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# Growth of the Photosynthetic Bacterium *Rhodopseudomonas capsulata* Chemoautotrophically in Darkness with $H_2$ as the Energy Source

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The phototrophic bacterium *Rhodopseudomonas capsulata* was found to be capable of growing chemoautotrophically under aerobic conditions in darkness. Growth was strictly dependent on the presence of $H_2$ as the source of energy and reducing power, $O_2$ as the terminal electron acceptor for energy transduction, and $CO_2$ as the sole carbon source; under optimal conditions the generation time was about 6 h. Chemoautotrophically grown cells showed a relatively high content of bacteriochlorophyll *a* and intracytoplasmic membranes (chromatophores). Experiments with various mutants of *R. capsulata*, affected in electron transport, indicate that either of the two terminal oxidases of this bacterium can participate in the energy-yielding oxidation of $H_2$. The ability of *R. capsulata* to multiply in at least five different physiological growth modes suggests that it is one of the most metabolically versatile procaryotes known.

Molecular hydrogen is a prominent substrate and end product in the anaerobic metabolism of numerous photosynthetic bacteria (3). Dihydrogen gas can be used as a source of electrons for photoautotrophic growth and is produced in large quantity during photoheterotrophic metabolism under certain conditions of nitrogen nutrition (3, 5). It has also been observed that resting cells of certain *Rhodospirillaceae* can catalyze the Knallgas reaction ($2\ H_2 + O_2 \rightarrow 2\ H_2O$), which represents the energy transduction system of various nonphotosynthetic "hydrogen" bacteria (14, 17). Because $H_2$ can be used as the electron source for reductive biosynthesis, as well as for the fuel for aerobic oxidative phosphorylation in the latter organisms, it seemed likely to us that some species of facultatively aerobic photosynthetic bacteria probably are capable of growing chemoautotrophically on $H_2$-$CO_2$-$O_2$ in darkness. *Rhodopseudomonas capsulata* warranted particular study because this bacterium grows readily on $H_2$ plus $CO_2$ with light as the energy source (6) and also shows a highly developed heterotrophic (dark) respiratory metabolism. The experiments described here demonstrate that, indeed, *R. capsulata* can grow as an aerobic chemoautotroph on $H_2$ in darkness and that cells multiplying in this fashion produce bacteriochlorophyll and an abundance of intracytoplasmic membranes. Thus, five growth modes are available to this remarkable bacterium: (i) anaerobic growth as a photoautotroph on $H_2$ plus $CO_2$ with light as the energy source; (ii) anaerobic growth as a photoheterotroph on various organic carbon sources with light as the energy source; (iii) growth as a fermentative anaerobe, in darkness, on sugars as sole carbon and energy sources; (iv) aerobic growth as an ordinary chemoheterotroph in darkness; and (v) aerobic growth as a chemoautotroph, in darkness, with $H_2$ as the source of electrons.

## MATERIALS AND METHODS

**Bacterial strains.** *R. capsulata* B10 was used as a representative wild-type strain (26) capable of growing photosynthetically under anaerobic conditions or aerobically in darkness as a chemoheterotroph. Mutants M4, M5, and M6 are respiratory-deficient derivatives of *R. capsulata* St. Louis that retain the capacity to grow photosynthetically (12). On the contrary, Y11 is a mutant of *R. capsulata* that can grow aerobically in darkness, but which is unable to use light as an energy source owing to a lesion that affects cyclic (light-dependent) electron transport (11). *Rhodopseudomonas sphaeroides* 2.4.1 and *Rhodospirillum rubrum* 1.1.1 were originally obtained from the culture collection of C. B. van Niel.

**Media.** The defined mineral salts (plus vitamins) medium (designated as CA) used for chemoautotrophic growth with $H_2$ as the sole energy source contained (per 970 ml of deionized water): 20 mg of $Na_2$-ethylenediaminetetraacetic acid; 12 mg of $FeSO_4 \cdot 7H_2O$; 1 ml of trace element solution [containing, per 250 ml of deionized water: 700 mg of $H_3BO_3$; 398 mg of $MnSO_4 \cdot H_2O$; 188 mg of $Na_2MoO_4 \cdot 2H_2O$; 60 mg of $ZnSO_4 \cdot 7H_2O$; 10 mg of $Cu(NO_3)_2 \cdot 3H_2O$]; 200 mg of $MgSO_4 \cdot 7H_2O$; 75 mg of $CaCl_2 \cdot 2H_2O$; 1 g of NaCl; 1

g of $(NH_4)_2SO_4$; 1 mg of thiamine-hydrochloride; and 15 μg of biotin, with the pH adjusted to 7.2 before autoclaving. Just before use, the foregoing (970 ml) was supplemented with 30 ml of a sterile solution containing 1.2 g of $KH_2PO_4$ and 1.8 g of $K_2HPO_4$ (pH 7.2). For chemoautotrophic growth tests with *R. sphaeroides*, medium CA was augmented with 1 mg of nicotinic acid per liter. Solid media were prepared by addition of 1% (wt/vol) Wilson Ionagar.

The medium for photoheterotrophic growth was either the standard malate plus $NH_4^+$ medium known as RCVB (23) or a modification in which malate was omitted and replaced with either lactate or pyruvate (sodium salts, added from filter-sterilized stock solutions to give a concentration of 30 mM).

A complex medium (YPS) was employed for determining colony-forming units and checking the purity of cultures. This contained, per liter of deionized water: 3 g each of yeast extract (Difco) and peptone (Difco), 493 mg of $MgSO_4 \cdot 7H_2O$, and 294 mg of $CaCl_2 \cdot 2H_2O$. For plates, the medium was solidified with 1.2% (wt/vol) agar (Difco).

**Growth conditions.** For chemoautotrophic growth in liquid culture, 250-ml or 2.5-liter Erlenmeyer flasks were used, containing 200 or 2,000 ml of CA medium, respectively, and a magnetic stirring bar. After inoculation (with cells grown photosynthetically in RCVB medium ± nicotinic acid; such inocula were also used for plate cultures), each flask was sealed with a rubber stopper fitted with two gas-sparging needles (one for $CO_2 + H_2$, the other for $O_2$) and a gas-exit needle. Cultures were continuously bubbled at a rate of 100 ml of gas per min with a mixture of $H_2$-$O_2$-$CO_2$ (85:10:5) as follows: $H_2$ and $CO_2$ gases (Linde Air Products Co.) were mixed in an appropriate ratio by using a gas rotameter, and the mixture was passed through a sterilized cotton filter and then through the culture; sterile $O_2$ was similarly introduced into the culture at a suitable flow rate (checked by measuring the rate of water displacement from a filled vessel). To prevent evaporation of medium in long-term experiments, the gases were initially passed through gas-washing bottles filled with distilled water. Flasks were incubated in a completely darkened aquarium water bath (32 to 34°C) placed on a magnetic stirrer; the stirring rate was about 250 rpm. Cells were also grown chemoautotrophically on agar plates incubated in GasPak anaerobic system jars (Bioquest) modified by removal of the palladium catalyst pellets. The GasPak $H_2$ plus $CO_2$ generator component (activated with 10 ml of 25% [wt/vol] $KH_2PO_4$ instead of water, to ensure maximal $CO_2$ evolution) was added as the source of gaseous substrates, other than $O_2$, and the jars were sealed as usual before incubation in darkness at 30°C.

Cultures were grown photoheterotrophically as described by Weaver et al. (26). For photoautotrophic growth (on $CO_2 + H_2$), 200 ml of inoculated CA medium was placed in a 1-liter flask, the culture was gassed with 20% $H_2$-5% $CO_2$-75% $N_2$, and the flask was sealed with a rubber stopper; during incubation the culture was constantly mixed by a magnetic stirrer (150 rpm). All photosynthetic cultures were grown at 35°C with a light intensity of 6,400 lx (lumiline incandescent lamps).

For chemoheterotrophic (dark) growth, cultures of a 25- or 50-ml volume in 125- or 250-ml Erlenmeyer flasks, respectively, were shaken (150 rpm) in a rotary-shaker water bath at 35°C; the medium was either RCVB or a modification with lactate replacing malate.

Agar plates used for estimation of colony-forming units and for checking culture purity were incubated aerobically in darkness at 30°C.

**Measurement of bacterial growth.** Four independent assays of bacterial multiplication were employed: (i) culture density, measured with a Klett-Summerson colorimeter fitted with a no. 66 filter (it was found that photometer readings and bacterial dry weight were proportional up to about 200 photometer units); (ii) dry weight of cells centrifuged from 10 ml of culture—pellets were washed once with deionized water and dried to constant weight (at 110°C) in preweighed aluminum-foil boats; (iii) cell protein—cell pellets were dispersed in 1 N NaOH, the suspensions were immersed in a boiling-water bath for 20 min, and, after centrifugation, protein contents of the supernatant fluids were determined by the procedure of Lowry et al. (8); (iv) increase in viable count (colony-forming units)—the plating procedure of Wall et al. was used (25).

**Absorption spectra.** The in vivo absorption spectrum of bacteriochlorophyll (BChl) in chemoautotrophically grown cells (suspended in about 30% [wt/vol] bovine serum albumin [19]) and the spectrum of BChl extracted into acetone-methanol (7:2) (2) were obtained by using a Cary model 14 spectrophotometer.

Membrane preparations, used for observing cytochrome spectra, were obtained from cells disrupted in a French pressure cell; the suspending medium was 50 mM glycylglycine buffer (pH 7.2). Crude extracts were centrifuged at 27,000 × *g* for 10 min to remove debris, and the highly pigmented supernatant fluid was then centrifuged at 144,000 × *g* for 90 min. The packed membrane fragments were resuspended in glycylglycine buffer, and the reduced-minus-oxidized difference spectra were determined over the range of 520 to 620 nm (Cary model 14 spectrophotometer); solid oxidant $[K_3Fe(CN)_6]$ and reductant (sodium ascorbate or $Na_2S_2O_4$) were added to the reference and sample cuvettes, respectively.

**BChl determination.** BChl was extracted from cell pellets with 100% methanol for 1 h at −20°C (in darkness). The suspensions were centrifuged to remove insoluble residue, and the absorbancy at 772 nm was measured in a Zeiss PMQ2 spectrophotometer. BChl concentrations were calculated by using an absorption coefficient of 46.1 liters/g per cm for BChl *a* in methanol (18).

**Chemical determinations.** Cells were analyzed for poly-β-hydroxybutyrate by the method of Law and Slepecky (7) and for total carbohydrate as described by Stanier et al. (20).

**Electron microscopy.** Cells were fixed by the method of Ryter et al. (16), dehydrated in an ethanol series, and embedded in Spurrs low-viscosity embedding medium. Sections cut with a diamond knife were stained with uranyl acetate and lead citrate and then examined with a Philips model 300 electron microscope operating at 60 kV.

## RESULTS

**Chemoautotrophic growth of *R. capsulata* in darkness with $H_2$ as the energy source.** The kinetics of chemoautotrophic growth of *R. capsulata* with $H_2$ as the sole energy source under aerobic conditions in darkness are shown in Fig. 1. Cells grown in this fashion show zig-zag chain formations, a well-known characteristic of *R. capsulata* observed in other growth modes (26). Each of the independent measures of bacterial growth increases as would be expected during exponential growth (up to about 20 h) (Fig. 1). Complete dependence of growth on the presence of $H_2$ is evident from the controls shown in which $H_2$ was replaced by $N_2$. In other control experiments, it was determined that omission of either $O_2$ or $CO_2$ from the gas mixture also completely prevented growth (data not shown). We conclude that *R. capsulata* can grow readily as an aerobic chemoautotroph in darkness; that is, with reducing power and energy derived from the oxidation of $H_2$. The controls showed that the individual gases employed did not contain sufficient organic contaminants to sustain appreciable aerobic chemoheterotrophic growth under the experimental conditions used.

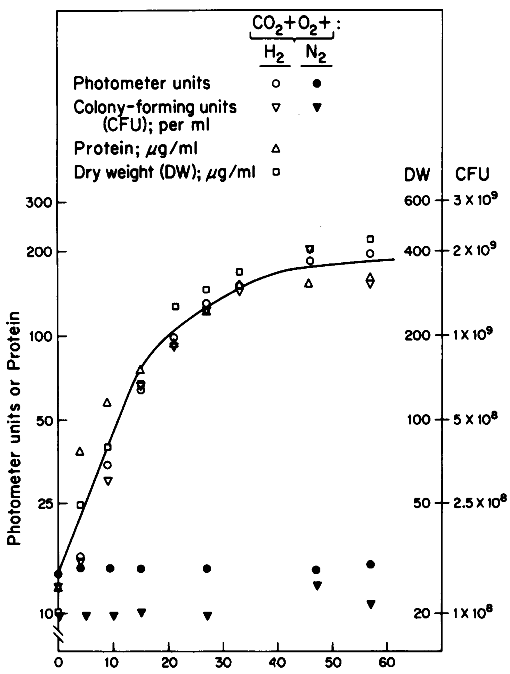


FIG. 1. *Growth of R. capsulata B10 chemoautotrophically in darkness on $H_2$-$CO_2$-$O_2$. Cells were grown in 2.5-liter flasks at 33°C as described in the text. Samples were removed periodically with a sterile syringe (via a stainless-steel needle mounted in the vessel stopper) for the measurements indicated. The gas atmospheres initially contained 5% $CO_2$ and 10% $O_2$ plus 85% $H_2$ (open symbols) or 85% $N_2$ (closed symbols). At 15 h, the gas mixtures were altered so as to contain either 70% $H_2$ or $N_2$-20% $O_2$-10% $CO_2$. Symbols: ○, Culture turbidity expressed in photometer units; ▽, colony-forming units (CFU) per milliliter; □, dry weight (DW; micrograms per milliliter); △, protein (micrograms per milliliter).*

Trials were conducted to determine the optimal concentration of $O_2$ for chemoautotrophic growth. Although low (ca. 5%) concentrations of $O_2$ permit growth, the maximal rate was observed with ca. 10% $O_2$. An initial $O_2$ concentration of 20% inhibited growth of cultures inoculated with low cell densities, but an increase from 10 to 20% $O_2$ during the mid-log phase of growth was stimulatory and prevented the culture from entering stationary phase prematurely. Oxygen concentrations higher than 20% completely inhibited growth regardless of culture density. Attempts to replace $O_2$ with nitrate or fumarate as terminal electron acceptors under anaerobic conditions gave negative results.

The exponential portion of the growth curve of Fig. 1 indicates a generation time of ca. 6 h under chemoautotrophic conditions. Essentially the same doubling time is observed for dark fermentative growth of *R. capsulata* on fructose as the sole energy source (Table 1). A comparison of these growth rates with those typically seen in the other three alternative growth modes available to *R. capsulata* is found in Table 1; substantially faster doubling times were observed under photoautotrophic, photoheterotrophic, and chemoheterotrophic conditions.

**Characteristics of chemoautotrophically**

TABLE 1. *Growth rates of R. capsulata B10*

| Growth mode[a] | Generation time (h) |
|---|---|
| Photoautotrophic (anaerobic): Gas phase, 20% $H_2$-5% $CO_2$-75% $N_2$ | 3.5 |
| Photoheterotrophic (anaerobic) | |
| C source: Malate | 2.0 |
| Lactate or pyruvate | 1.8 |
| Chemoheterotrophic (aerobic; dark) | |
| C source: Malate | 2.8 |
| Lactate | 1.8 |
| Chemoautotrophic (aerobic; dark) Gas phase, 85% $H_2$-5% $CO_2$-10% $O_2$ | 6.0 |
| Fermentative (anaerobic; dark): 20 mM fructose serving as sole C and energy source (in presence of 30 mM trimethylamine-*N*-oxide)[b] | 6.0 |

[a] Growth conditions as detailed in the text.
[b] For a description of this growth mode see reference 10.

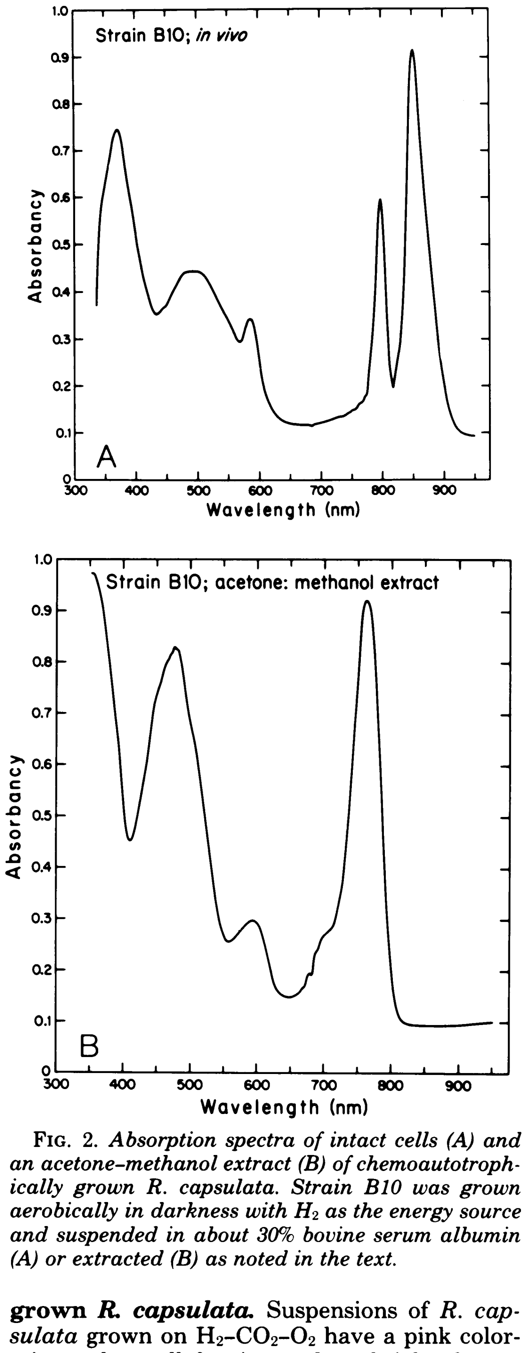


FIG. 2. *Absorption spectra of intact cells (A) and an acetone-methanol extract (B) of chemoautotrophically grown R. capsulata. Strain B10 was grown aerobically in darkness with $H_2$ as the energy source and suspended in about 30% bovine serum albumin (A) or extracted (B) as noted in the text.*

**grown *R. capsulata*.** Suspensions of *R. capsulata* grown on $H_2$-$CO_2$-$O_2$ have a pink coloration at low cell density, and are bright cherry-red at high cell concentration. An in vivo absorption spectrum of chemoautotrophically grown cells is shown in Fig. 2A. Peaks in the infrared at about 800 and 855 nm clearly indicate the presence of BChl *a*. Resolution of carotenoid pigment maxima in the region of 425 to 500 nm is not evident in Fig. 2A. However, acetone-methanol extracts of cells (Fig. 2B) show one major peak at 480 nm, characteristic of carotenoids of the "alternative spirilloxanthin series"; these are the major carotenoids normally found in *R. capsulata* (26).

The specific BChl content of chemoautotrophically grown cells of *R. capsulata* is quite high; namely, about 25 to 30 $\mu$g of BChl *a* per mg of cell protein; cells grown photosynthetically at saturating light intensity (6,400 lx) usually show values of 15 to 20. The high specific BChl content of cells grown on $H_2$-$CO_2$-$O_2$ correlates well with an abundance of intracytoplasmic membranes (chromatophores) in such cells (Fig. 3A); in comparison, cells grown photosynthetically at high light intensity show relatively few chromatophores (Fig. 3B). Although large clear areas usually characteristic of storage materials are frequently seen in electron micrographs of chemoautotrophically grown *R. capsulata* (Fig. 3A), we have detected relatively small quantities of poly-$\beta$-hydroxybutyrate (<1% of the cell dry weight) and glycogen (ca. 3% of the cell dry weight).

Reduced-minus-oxidized difference spectra of membranes of chemoautotrophically grown cells showed absorption maxima at 553 and 561 nm, indicating cytochromes of the *c* and *b* types, respectively. The *c*-type component is presumably the high midpoint potential cytochrome $c_2$ known to be involved in both respiratory and photosynthetic electron flow (12). The 561-nm peak, however, could be due to any one, or more, of the several *b*-type cytochromes known to be synthesized by *R. capsulata* under various growth conditions (29).

**Chemoautotrophic growth of respiratory and photosynthetic mutants of *R. capsulata*, and of related organisms.** Table 2 summarizes results of growth tests with wild-type *R. capsulata* and several mutants with blocks in functional oxidase activities (i.e., blocks affecting an oxidase itself or a closely associated electron transport step). The results clearly indicate that functional oxidase activity is required for chemoautotrophic growth. Growth on $H_2$-$CO_2$-$O_2$ occurs, but is significantly slower in mutants lacking functional cytochrome $b_{410\ \mathrm{mV}}$ oxidase activity. Electron transport to this oxidase is mediated by cytochrome $c_2$ and is known to result in a higher ATP yield than electron flow to the alternative cytochrome $b_{260\ \mathrm{mV}}$ oxidase (1).

Tests for chemoautotrophic development of *R. rubrum* 1.1.1 on agar suggested limited ability to grow in this mode, as indicated by very small colony size (appearance of colonies, however, was dependent on the presence of $H_2$). Preliminary experiments with *R. rubrum* 1.1.1 and *R. sphaeroides* 2.4.1 using liquid cultures and an atmosphere of $H_2$-$CO_2$-$O_2$ gave inconclusive

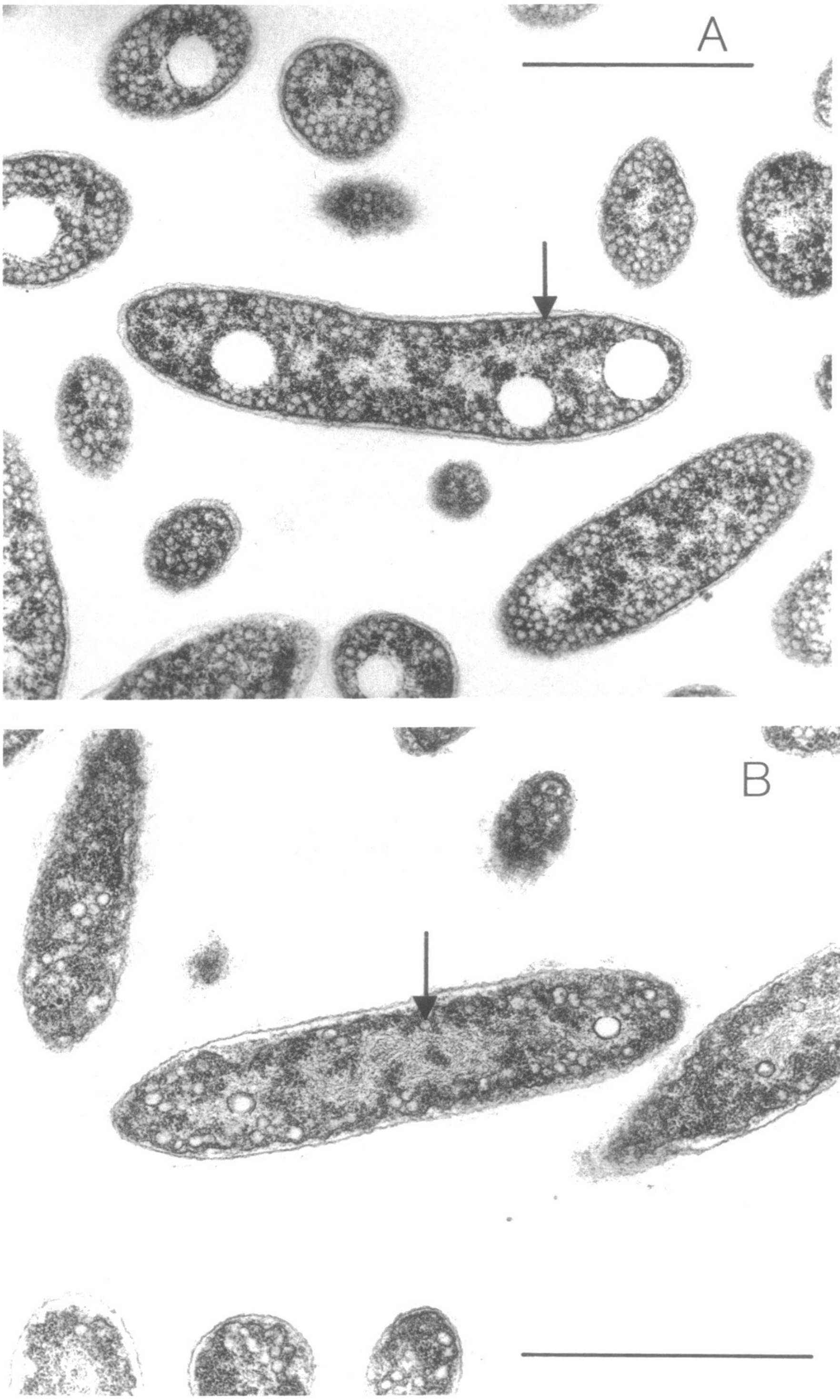


FIG. 3. *Electron micrographs of thin sections of R. capsulata B10 grown chemoautotrophically in darkness with* $H_2$ *(A) and photosynthetically (B). (B) Cells were grown anaerobically at saturating light intensity (6,400 lx). Note that the content of intracytoplasmic membrane (chromatophores, see arrows) is greater in the cells grown chemoautotrophically. Bar, 1 μm.*

TABLE 2. *Capacities of R. capsulata wild type and electron transport mutants to grow chemoautotrophically*

| Strain | Functional oxidase activity[a] | | Growth on $H_2$-$CO_2$-$O_2$[b] |
|---|---|---|---|
| | Cytochrome $b_{260\ mV}$ | Cytochrome $b_{410\ mV}$ | |
| B10 (wild type) | Present | Present | ++ |
| M4 | Present | Absent | + |
| M5 | Absent | Absent | − |
| M6 | Absent | Present | ++ |
| Y11 | Present | Absent | + |

[a] For further details on characteristics of the alternative oxidase systems see references 28 and 29; the nature of the Y11 defect is discussed by Marrs (11).

[b] Colony diameter on agar plates after 1 week of incubation at 30°C in darkness: ++, 1 to 3 mm; +, <1 mm; −, no growth.

(that is, essentially negative) results in both instances.

## DISCUSSION

Results from earlier studies (6) suggest that among *Rhodospirillaceae* species the capacity to grow photoautotrophically on $H_2$ + $CO_2$ is particularly well-developed in *R. capsulata*. Since the reductive pentose cycle is undoubtedly the major pathway for light-dependent autotrophic $CO_2$ assimilation with $H_2$ in this bacterium (4, 21), it is reasonable to expect that *R. capsulata* can utilize this pathway to support growth with an alternative mode of energy generation, namely, the oxidation of $H_2$ with $O_2$. Gibson and Tabita (4) have detected two forms of ribulose-1,5-bisphosphate carboxylase in photosynthetically grown cells of *R. capsulata* which differ in respect to molecular weight, pH optimum, and response to the effector 6-phosphogluconate. It appears that ribulose-1,5-bisphosphate carboxylases from all sources, including photosynthetic bacteria (9), have a potential alternative enzymatic activity, namely, an oxygenase function that produces phosphoglycolate from ribulose-1,5-bisphosphate. Accordingly, it is possible that comparative studies of the enzyme(s) from *R. capsulata* cells grown in anaerobic-photoautotrophic and aerobic-chemoautotrophic modes may provide valuable insights into its regulatory properties.

The behavior of *R. capsulata* in regard to optimal $O_2$ concentration for chemoautotrophic development resembles that observed with several non-photosynthetic $H_2$-oxidizing bacteria (Knallgas bacteria; 15, 17). During the initial stages of growth, at low cell density, a diminished $O_2$ tension is favorable; on the other hand, as the bacterial density increases, greater concentrations of $O_2$ are tolerated and, in fact, stimulate growth. Different species of $H_2$-oxidizers have been reported to differ in their tolerance to $O_2$; some require much less than 10% $O_2$ for optimal growth, whereas others are relatively insensitive to very high $O_2$ concentrations (15). Inhibitory effects of high $O_2$ tension are usually attributed to repression of synthesis and/or inhibition of activity of hydrogenase. Such effects are likely to be a major cause of $O_2$ inhibition of chemoautotrophic growth of *R. capsulata* and of *Rhodopseudomonas acidophila*; the capacity for aerobic chemoautotrophic growth of the latter with $H_2$ was recently noted by N. Pfennig and E. Siefert (Abstr. Int. Symp. Microb. Growth on C-1 Compounds, Puschino, U.S.S.R, 1977, p. 146). The hydrogenase referred to is the so-called "uptake" or "conventional" hydrogenase, the enzyme that activates $H_2$ for use as a biosynthetic reductant or as a source of electrons for energy transduction. Under certain conditions, *R. capsulata* and related bacteria produce $H_2$ by a light-dependent process in which nitrogenase catalyzes proton reduction to dihydrogen gas (3, 5). As far as is known, nitrogenase cannot activate $H_2$ in the fashion indicated for "conventional" hydrogenase. In fact, certain mutants of *R. capsulata* unable to produce $H_2$ due to defects in nitrogenase retain the capacity to grow normally on $H_2$ + $CO_2$ under anaerobic, photosynthetic conditions (24). This illustrates the independence of the $H_2$ uptake and $H_2$ production systems in *R. capsulata* (5, 24).

It has been apparent for some time that certain representatives of the *Rhodospirillaceae* have unusually great metabolic capacities (13, 22). Thus, *R. capsulata* was known to grow readily anaerobically as a photoautotroph, as a photoheterotroph, or as an aerobic chemoheterotroph (6, 22). In addition, this organism can use a remarkably wide range of nitrogen sources for growth, including $N_2$, $NH_4^+$, urea, various amino acids, etc. (23). With the recent discovery that *R. capsulata* can also grow anaerobically in darkness on sugars as sole carbon and energy sources by an unusual fermentation process (10, 27), its exceptional versatility in respect to alternative modes of energy transduction was further emphasized. The capacity of *R. capsulata* to grow aerobically in darkness as a chemoautotroph with $H_2$ as the energy source represents the fifth distinct growth mode demonstrated for this bacterium and makes *R. capsulata* unique among known organisms.

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